AD_____

AWARD NUMBER DAMD17-97-1-7152

TITLE: Immunotherapeutic Cell-Based Vaccine to Combat Metastatic Breast Cancer

PRINCIPAL INVESTIGATOR: Beth A. Pulaski

CONTRACTING ORGANIZATION: University of Maryland Baltimore County
Baltimore, Maryland 21250

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE

Immunotherapeutic Cell-Based Vaccine to Combat Metastatic Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7152

6. AUTHOR(S)

Beth A. Pulaski, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Maryland Baltimore County
Baltimore, Maryland 21250

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19981210 099

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)* For many cancer patients removal of the primary tumor is curative; however, if metastatic lesions exist and are not responsive to treatment, survival is limited. Although immunotherapy is actively being tested in animal models against primary tumors and experimental metastases (i.v. induced) very few studies have examined immunotherapy of spontaneous, established metastatic disease. The shortage of such studies can be attributed to the paucity ofadequate animl models and to the concern that multiple metastatic lesions are more resistant to immunotherapy than a localized primary tumor. In this report, we use the BALB/c-derived mouse mammary carcinoma, 4T1, and show that this tumor very closely models human breast cancer in its immunogenicity, metastatic properties, and growth characteristics. Therapy studies demonstrate that treatment of mice with established primary and metastatic disease with MHC class II and B7.1 transfected tumor cells reduces or eliminates established spontaneous metastases but has no impact on primary tumor growth. These studies indicate that cell-based vaccines targeting the activation of CD4+ and CD8+ T cells may be effective agents for the treatment of malignancies, such as breast cancer, where the the primary tumor is curable by conventional methods, but metastatic lesions remain refractile to current treatment modalities.

14. SUBJECT TERMS

Breast Cancer, Immunotherapy, Metastases, CD4+ and CD8+ T-lymphocytes, 4-1BB-L Co-stimulation, SEB Superantigen

15. NUMBER OF PAGES 32

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

BP Where copyrighted material is quoted, permission has been obtained to use such material.

BP Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

BP Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

BP In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

BP In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

BP In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

BP In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Bethel Pulaski 7/28/98

PI - Signature Date

Table of Contents

Introduction

Stimulating immune responses to tumors poses an interesting challenge. Tumor cells are not very potent immunogenic agents as they typically lack the surface receptors needed to induce immune responses. For example, tumors can lack class I (1-3) and/or class II (4) major histocompatibility complex (MHC) gene products which are needed for presentation of antigen to $CD8^+$ and $CD4^+$ T-cells, respectively (5, 6). Likewise, co-stimulatory molecules are often not expressed on tumors and have been shown to be important for induction of tumor-specific immunity (7). Thus, it has been postulated that poor immune responses to tumors are caused by immunological "ignorance", meaning that the immune system has the capacity to respond to tumor antigens presented by tumor cells but fails to respond under normal conditions (8).

Recent advances in immunotherapy have led to the generation of protective T-cell-mediated immunity against primary tumors. The stimulation of $CD8^+$ cytotoxic T-lymphocytes (CTL) has been the primary focus of many studies as these effector cells are capable of specifically and directly destroying malignant tumor cells. With our increasing knowledge regarding immune response mechanisms and with the isolation/identification of genes involved in CTL activation, these groups have begun to manipulate the immune system to their advantage by designing tumor cells to act as better antigen presenting cells (APC). For example, various cytokine genes have been transfected or transduced into tumors and have enhanced immune responses to primary tumors (9, 10). Interestingly, while it was intended to circumvent the need for $CD4^+$ T-cell help by allowing the tumor cells to directly supply cytokines to CTL, some studies demonstrated that both $CD4^+$ and $CD8^+$ T-cells were necessary (11-13).

Methods for effective treatment of metastatic disease still need to be developed. While advances have been made to increase the immunogenicity of tumors, work still needs to be done to create effective tumor vaccines that eliminate tumor cells found in sites other than at the primary tumor. In some cases, such as in human breast cancer where the primary tumor has not metastasized, surgical removal of primary tumors can lead to full recovery of the patient. However, when the primary breast tumors have metastasized, other therapies such as chemotherapy are needed to eliminate metastatic tumor cells and may not always be successful. Therefore, by designing tumor

cells to act as the vaccination vehicle for stimulating both $CD4^+$ and $CD8^+$ T-cells, it would be possible to induce long-lived, tumor-specific responses that can be used in combination with established methods.

Optimal T-cell activation requires an antigen-specific signal plus a second co-stimulatory signal. T-cells recognize peptide/MHC complexes through their T-cell receptor (TCR) (14,15). However, to achieve maximum activation of $CD4^+$ or $CD8^+$ T-cells, a second TCR-independent signal (co-stimulation) is required (16). Several studies have demonstrated the role of B7.1 and B7.2 in co-stimulation (17). However, other molecules, such as ICAM-1, VCAM-1, and heat stable antigen, can also function in a co-stimulatory fashion (18-20). Interestingly, 4-1BB ligand (4-1BB-L), a newly described molecule found on activated macrophages and mature B cells, also enhances proliferative T-cell responses and synergizes with B7 (21-24). Ultimately, failure of T-cells to receive both signals from APC will result in T-cell anergy (unresponsiveness) or death.

Designing tumor cells to act as the APC is a feasible rationale. Using tumor cells as APC is advantageous as relevant tumor antigen will be presented to T-cells. With the right combination of genes expressed by tumor cells, these APC can possess an increased immunogenicity and subsequently stimulate immune responses. For example, our previous studies have shown that the transfection of class II MHC into sarcoma and melanoma cells enhanced tumor rejection and reduced metastatic potential (25), respectively. Furthermore, expression of either B7.1 or B7.2 in addition to class II MHC increased these effects (25, 26). We would like to broaden our studies to include genes, such as 4-1BB-L and *S. aureus* enterotoxin B (SEB), which demonstrate significant potential to synergize with our existing approach.

4-1BB-L can function as a co-stimulatory molecule. 4-1BB-L, a member of the TNF receptor family, is a recently discovered ligand for the 4-1BB co-stimulatory molecule (21). This molecule holds great potential as previous studies suggest its importance in co-stimulation of IL-2 production and proliferation by T-cells (21-23). While it is not certain that this molecule alone will enhance the immunogenicity of tumor cells, it is possible that 4-1BB-L will synergize with other receptors. Studies have shown that the B lymphoma M12 expresses low levels of 4-1BB-L but is induced by treatment with cAMP which also induces B7.1 and B7.2 (22). Therefore, this may suggest a synergistic effect of these co-ordinately-controlled co-stimulatory molecules.

Subsequently and if needed, transfectants co-expressing 4-1BB-L, B7.1, and/or class II MHC will be made as these signals may synergize.

SEB superantigen (sAg) can activate $CD4^+$ T-cells in a polyclonal manner. The toxin SEB is a well-known sAg that has been established as a potent stimulator of T-cells when complexed with class II MHC (27). Therefore, transfectants expressing both class II and SEB hold good potential for stimulating tumor-specific T-cells. However, the potential of this combination relies on the fact that SEB activates T-cells which express TCR containing $V_\beta 7$ or $V_\beta 8$ gene segments (28). This may not be a significant problem as administration of SEB either *in vivo* or *in vitro* has been shown to activate T-cells against the 1591 skin tumor (29) and the MCA 205/207 sarcomas (30), respectively. Recently, it was also demonstrated that pulmonary metastases formed by CL-62 melanoma cells injected intravenously can be neutralized using soluble SEB (31). In our strategy, where the tumor cells would directly express SEB, activation of T-cells would occur more efficiently (smaller amounts of SEB would be needed to stimulate its effects) and would less likely cause adverse side effects sometimes associated with large systemic treatments.

The mouse 4T1 mammary carcinoma is an excellent tumor for studying metastatic disease. The tumor system chosen for these studies is 4T1, the poorly immunogenic mammary tumor (32-34). This tumor shares many characteristics with human mammary cancers and expresses adequate levels of MHC class I MHC, making it an excellent animal model and a suitable target for $CD8^+$ T cells. Because 4T1 is 6-thioguanine resistant, micrometastatic cells can be readily detected at very early stages of growth, allowing us to quantitatively monitor the effects of the immunotherapy approach on spontaneous metastasis development.

Body

Hypothesis/Purpose

In breast cancer patients, primary tumor burden seldom causes the patient's demise as primary tumor can be surgically removed. In fact, it is spontaneous metastases that arise during the tumor growth or long after the primary tumor has been removed to which the patient eventually succumbs. Therefore, the purpose of this proposal is to design a mammary tumor vaccine using the tumor cells themselves to directly activate the immune system to combat spontaneous metastatic disease either before or after it becomes established. In particular, we will engineer the 4T1 mammary tumor cells to express class II MHC, 4-1BB-L, and/or SEB with the expectation that these cells will activate $CD4^+$ T-cells and subsequently $CD8^+$ T-cells, immune effectors capable of circulating throughout the body to destroy metastatic tumor cells specifically and provide long term memory.

Technical Objective 1: Generate mammary tumor transfectants which can more effectively and directly present tumor antigen to $CD4^+$ T-cells.

Task 1: cDNA Expression vectors. Genes such as class II MHC ($I\text{-}A_\alpha^d$, $I\text{-}A_\beta^d$), 4-1BB-L, B7.1, and SEB have been subclonded into the multiple cloning site of the pHβ-Apr-1-neo expression vector, previously described (35) (Figure 1). Using the polymerase chain reaction (PCR), cDNAs encoding the A_α^d and A_β^d class II MHC and 4-1BB-L genes were amplified from RNA isolated from A20 B-lymphoma cell line. The SEB gene was supplied by Dr. Saleem Kahn (University of Pittsburgh). The expression vector containing B7.1 was generated prior to this grant (36).

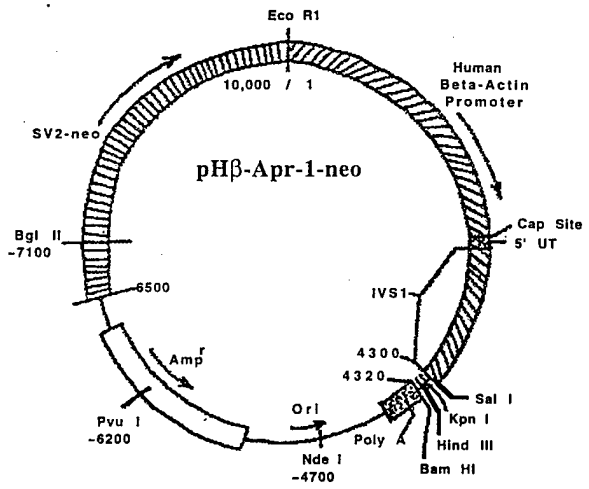


Figure 1: The pHβ-Apr-1-neo expression vector. This vector contains a human β-actin promoter and a neomycin selectable marker.

Tasks 2-3: Transfection of 4T1 cells. Single transfectants (4T1/A^d, 4T1/B7.1, 4T1/SEB, 4T1/4-1BB-L) and double transfectants (4T1/A^d.B7.1) were generated using the expression vectors described in task 1. Expression of MHC class II (A^d), and B7.1 was determined by immunofluorescence (Figure 2). These transfectants express

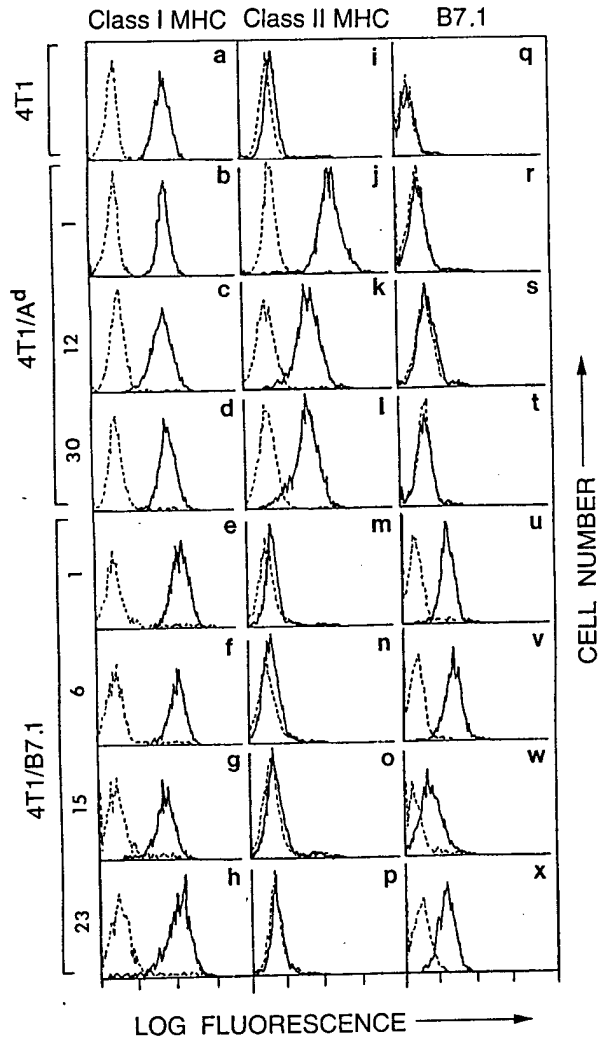
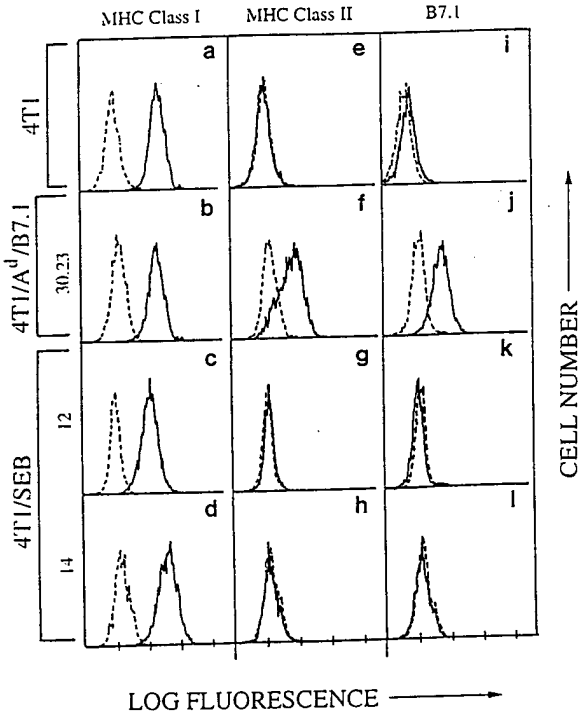


Figure 2: Transfectants were made with lipofectin following manufacturer's instructions, selected with 400 μg/ml G-418, cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry as described previously (25, 26). Class I MHC expression (a-h) was measured using the mouse anti-H-2D^d mAb 34-5-8 (37) (—) and irrelevant control mouse anti-H-2K^k mAb 16.3.1 (38) (····). Class II MHC expression (i-p) was measured using the mouse anti-A^d mAb MKD6 (39) (—) and the isotype matched irrelevant control mouse anti-A^{b,k} mAb 3JP (40) (····). B7.1 expression (q-x) was measured using the rat anti-B7.1 mAb 1G10 (41) (—) with the conjugate alone (····) as control. The x-axis shows four log cycles of fluorescence intensity.

similar levels of MHC class I as compared to parental 4T1 cells (Figure 2, panels a-h). Two of the MHC class II transfectant clones (4T1/A^d-12 and 4T1/A^d-30) express similar levels of MHC class II, while the third class II transfectant (4T1/A^d-1) expresses higher levels (Figure 2, panels j-l). Of the four B7.1 transfectants, two clones (4T1/B7.1-1 and 4T1/B7.1-6) express similar levels of B7.1 which are slightly higher than the levels expressed by the two other transfectants (4T1/B7.1-15 and 4T1/B7.1-23 (Figure 2, panels u-x). 4T1 cells transfected with the empty parental vector (4T1/neo)

do not express either MHC class II or B7.1 (data not shown), as observed with untransfected 4T1 cells (Figure 2, panels i, q). These same analyses were performed on the 4T1/A^d.B7.1 double transfectants and showed similar results (Figure 3). Unfortunately, due to difficulties with reagent reliability, it was not possible to screen 4T1 transfectants for expression of 4-1BB-L. staining B-cell lines (A20 or 2PK3), which are known to express 4-1BB-L, would not stain positive with the detection reagent (4-1BB-Ig) supplied by Immunex after several attempts (data not shown). Therefore, expression of 4-1BB-L can not be assessed at this time. However, the cell lines were placed in cryogenic storage until such a time when they can be re-evaluated. SEB expression by 4T1 was determined by culturing naïve spleen cells in supernatants generated from potential transfectants and measuring spleen cell proliferation (Figure 4). The class I MHC expression by the 4T1/SEB clones was measured as described above and likewise express similar levels as compared to parental 4T1 (Figure 3). Double transfectants expressing both MHC class II and SEB still need to be generated.

Figure 3: Analysis of MHC and B7.1 expression by 4T1/SEB and 4T1/Ad.B7.1 transfectants. Class I MHC (a-d), class II MHC (e-h) and B7.1 expression (i-l) were measured and depicted below as described in Figure 2.



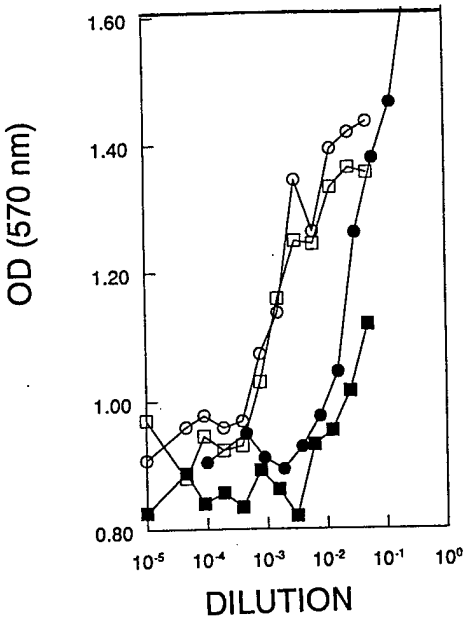


Figure 4: (above) Supernatants from 4T1/SEB transfectants stimulate proliferation of naïve spleen cells. Spleen cells were co-cultered for 3 days in the presence of either purified SEB (2mg/ml) (●), or supernatants collected from parental 4T1(■), 4T1/SEB-12 (□), and 4T1/SEB-14 (○). Proliferation was measured using MTT and read at OD 570nm.

In summary, all necessary expression vectors are completed and transfected into 4T1 cells with the following results: 3 out of 59 clones expressed class II MHC, 6 out of 10 clones expressed B7.1 and 4 were chosen for further study based on their expression patterns, 2 of the 3 class II^+ clones ($4T1/A^d$-1 and $4T1/A^d$-30) were double transfected with B7.1, and 2 out of 12 clones expressed SEB. Other transfectants are still needed, such as 4T1/4-1BB-L and $4T1/A^d$.SEB and will be generated at a later time. All of the transfectants created thus far demonstrated stable expression of the various genes and are systematically being used in the following technical objectives.

Technical Objective 2: Characterize the immunogenicity of the mammary tumor transfectants.

Tasks 4-5: *In vivo* kinetics of tumor growth and spontaneous metastases for 4T1 parental tumor. Previous studies by Miller and colleagues (32-34) established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. Since we are developing immunotherapy strategies for the treatment of metastatic malignancies, we have confirmed these results and assessed metastatic disease in additional target organs as a prelude to our therapeutic studies. As shown in Figure 5 and Table 1, primary tumors form in 100% of BALB/c mice when as few as $5x10^3$ cells are injected s.c. in the abdominal mammary gland. These tumors are palpable within 11-26 days after injection and reach 14-16 mm in size within 40-69 days. At higher doses ($>10^4$), primary tumors develop more rapidly as reflected in a shortened tumor onset and decreased survival time. While inoculation of lower doses of 4T1 (10^3) also induces primary tumor formation, the tumor incidence decreases to 60% of inoculated mice. The 4T1 tumor, therefore, is highly tumorigenic, even at relatively low doses of inoculating cells.

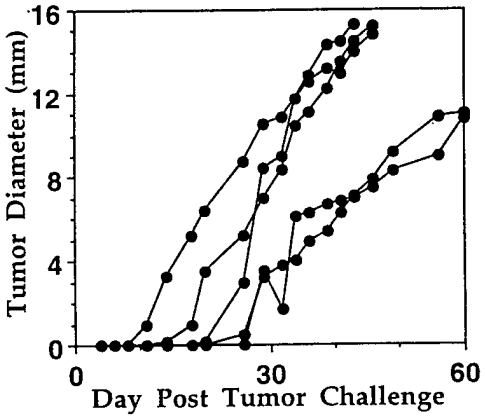


Figure 5: 4T1 cells are highly tumorigenic. Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 cells. Primary tumors were measured every 3-4 days and the mean tumor diameter (TD) was calculated as the square root of the product of two perpendicular diameters. Each line represents an individual mouse.

Table 1: *In vivo* Tumor growth analysis of 4T1 mammary carcinoma. BALB/c mice (5 mice/group) were challenged s.c. in the abdominal mammary gland with the indicated number of parental 4T1 tumor cells. The tumor incidence is the number of animals which developed progressive tumors. Animals which developed tumors were sacrificed when the TD reached 14-16 mm or when the mice became moribund.

Challenge Dose	Tumor Incidence	Tumor Onset (days)	Time to Sacrifice (days)
$1x10^3$	3/5	15-20	45-61
$5x10^3$	5/5	11-26	40-69
$1x10^4$	5/5	8-10	35-46
$1x10^5$	5/5	6-8	35
$1x10^6$	5/5	4-7	30

To confirm the metastatic potential of the 4T1 mammary carcinoma, female BALB/c mice were injected s.c. in the abdominal mammary gland with $5x10^3$ 4T1 cells and metastasis formation was assessed. Mice were sacrificed at varying times after inoculation and the kinetics of spontaneous metastasis formation were assessed in the draining lymph node (LN), lung, liver, blood, and brain by plating out dissociated organs in medium supplemented with 6-thioguanine. Because 4T1 cells are 6-thioguanine resistant, individual tumor cells form foci in culture, each focus representing an individual clonogenic tumor cell. The number of foci, therefore, is a direct measure of the number of metastatic tumor cells per organ and the *in vitro* amplification allows for the quantitation of micro-metastatic tumor cells which would otherwise not be detectable.

Table 2 shows the distribution and subsequent spread of metastatic tumor cells in the various organs at progressive times after inoculation. For example, at day 14 or 18 after primary s.c. inoculation, distant spontaneous metastases were measurable in the LN of 11/12 mice and the lungs of 13/13 mice. By day 22, the livers of 3/5 mice had clonogenic metastases, while the blood of only 1/8 mice contained tumor cells. Since only a portion of the blood was recovered, this value may be an under estimate. By week 4, the blood, liver, and lungs of 75 -100% of mice contained tumor cells. Some of the organs with clonogenic tumor cells showed visible metastatic lesions, however, many of the organs appeared phenotypically normal and showed no visible signs of tumor. Also by week 4, the draining LN of 5/8 mice had been engulfed by the primary tumor and, thus, could not

be tested. Metastatic cells in the brain were first detected at week 5 (27% of mice) and the frequency of mice with metastatic cells in the brain increased (67%) as time progressed. Metastases in the blood, LN, liver, and/or brain of individual mice were only present when the individual contained lung metastases, and not vice versa. The pathway of metastasis for the 4T1 tumor, therefore, appears to be from the primary tumor to the lungs and the draining LN and subsequently to the liver, blood and brain.

Table 2: 4T1 mammary carcinoma cells spontaneously metastasize in BALB/c mice.

Harvest Day	Spontaneous Metastases				
	Lymph Node	**Lung**	**Liver**	**Blood**	**Brain**
14-18	11/12 (2-57)	13/13 (1-43)	0/11	0/13	ND
22	7/9 (5-35)	6/11 (32-338)	3/5 (1)	1/8 (1)	ND
30-32	2/3 (15-83)	10/10 (6-116,500)	7/8 (7-3,700)	3/4 (6-82)	ND
34-37	ND	10/12 (315-267,000)	11/14 (32-7,800)	5/11 (1-24)	3/11 (1-116)
>42	ND	14/14 (1109-200,000)	6/8 (1,100-12,200)	6/8 (25-490)	4/6 (5-613)

BALB/c mice were challenged s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 tumor cells. Mice were sacrificed at various times after tumor challenge and the draining lymph node, lung, liver, blood, and brain tissues were removed. Each organ was individually prepared and plated for metastatic cell outgrowth (42). Data indicate the number of animals positive for spontaneous metastases out of the total number tested for each organ. The numbers in parentheses show the range of clonogenic metastases found in the positive organs. ND, not done.

There is frequently a correlation in human disease between the size of primary tumor and extent of metastatic disease. To determine if this observation is modeled by the 4T1 tumor, the number of clonogenic tumor cells in the lung, liver, blood, LN, and brain has been plotted as a

function of the tumor diameter (TD) at the time of harvest. As shown in Figure 6A, there is a positive correlation (correlation coefficient (cc): 0.684) between size of primary tumor at time of sacrifice and the number of clonogenic lung metastases. Similar correlations between TD at the time of harvest and clonogeinic metastases were also seen for liver (Fig 6B, cc: 0.520), blood (Fig 6C, cc: 0.396), and brain (Fig 6D, cc: 0.426). No correlation was seen between the number of clonogenic metastases in LN and the size of primary tumor (Fig 5E, cc: 0.134) as the number of samples were limiting. The 4T1 tumor, therefore, shows a pattern of metastatic spread comparable to human mammary carcinoma and assessment of lung metastases best approximates the extent of metastatic disease in tumor-bearing mice.

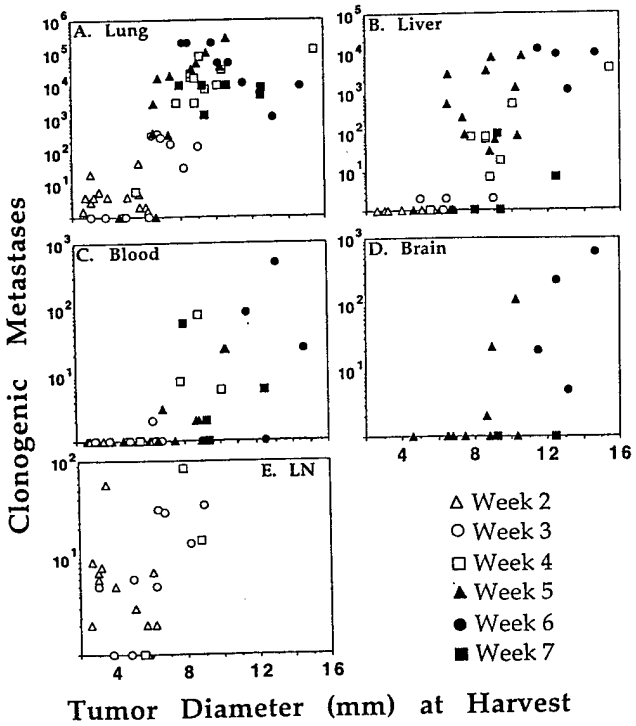


Figure 6: 4T1 tumor cells spontaneously metastasize to the lungs (A), liver (B), blood (C), brain (D), and LN (E). Syngeneic BALB/c mice were injected s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 cells. Mice were sacrificed at varying times after inoculation (weeks 2 to 7) and the number of metastatic tumor cells in the lungs determined as described in the Materials and Methods. Each point represents an individual mouse.

Tasks 6-7: *In vivo* tumor growth and spontaneous metastases development of 4T1 tumor single transfectants generated in task 2. To test tumorigenicity and spontaneous metastases development of the 4T1/A^d, 4T1/B7.1, and 4T1/SEB single transfectants, syngeneic female BALB/c mice were challenged in the abdominal mammary gland with $5x10^3$ transfectant cells. Figures 7 and 8 show the number of clonogenic tumor cells in the lungs vs. TD at time of sacrifice (large panels), and the growth rate of the primary tumor (inset panels) for the various transfectants. With the exception of 4T1/A^d-30 (Figure 7D, inset panel), all of the transfectants show some reduction in

primary tumor growth rate and/or lack of tumorigenicity, although, 4T1/A^d-12 and 4T1/SEB-12 transfectants do not form primary tumors in any of the inoculated mice (Figure 7C inset panel and Figure 8B inset panel, respectively). In contrast, the metastatic potential of the class II$^+$ or B7.1$^+$ or SEB$^+$ transfectants is markedly reduced relative to 4T1 cells. For example, 17/21 mice inoculated with class II$^+$ transfectants contained <5,000 metastatic cells in the lung (Figure 7B-D, large panels), while 15/15 mice inoculated with wild type 4T1 cells have 5,000 to 120,000 metastatic

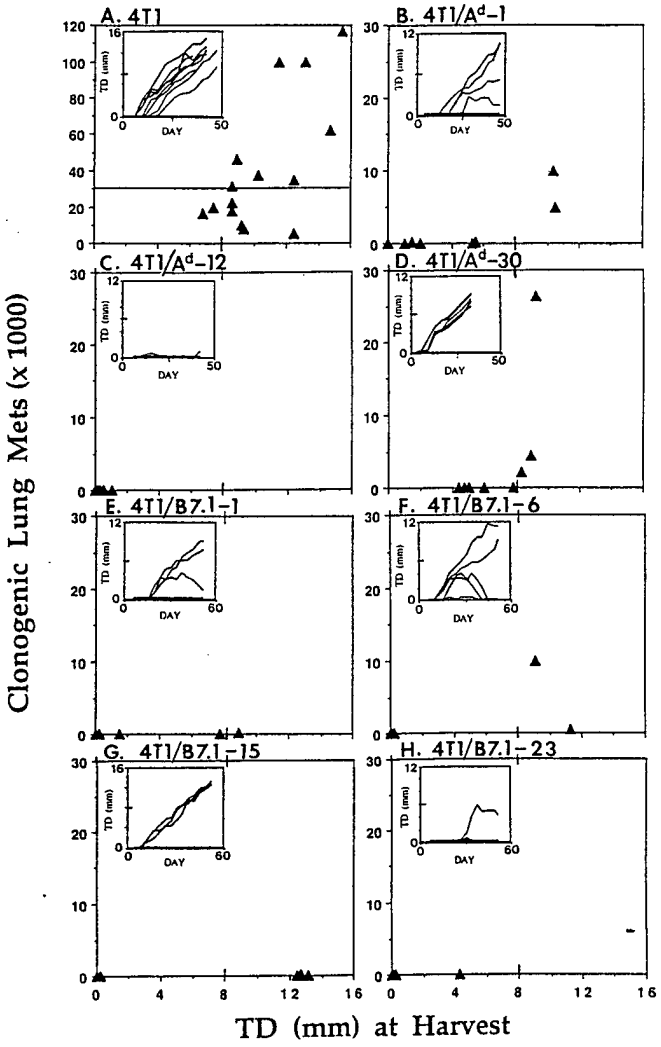


Figure 7: Expression of either class II MHC or B7.1 reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice were injected s.c. in the abdominal mammary gland with 5x10^3 parental 4T1 cells (15 mice) (A), 4T1/A^d-1 (9 mice) (B), 4T1/A^d-12 (10 mice) (C), 4T1/A^d-30 (8 mice) (D), 4T1/B7.1-1 (5 mice) (E), 4T1/B7.1-6 (5 mice) (F), 4T1/B7.1-15 (5 mice) (G), or 4T1/B7.1-23 (5 mice) (H) and sacrificed 32 to 55 days later and the number of metastatic cells in the lungs. Primary tumors were measured every 3-4 days. The larger panels show the number of clonogenic lung metastases (x 1000) versus TD at the time the mice were sacrificed. Each triangle represents an individual mouse. The smaller inset graphs show mean TD (y-axis) vs. day post inoculation (x-axis). Each line represents an individual mouse. Note that the number of clonogenic lung metastases shown on the y-axis ranges from 0-120 in panel A, as opposed to 0-30 for panels B-H.

cells in the lung (Figure 7A, large panel). For the B7.1^{+} transfectants, 19/20 inoculated mice contained 0 to 432 metastatic cells, with only one mouse displaying >10,000 tumor cells in the lungs (Figure 7E-H, large panels). In 8/10 mice inoculated with SEB^{+} transfectants, the lungs contained 0 metastatic cells (Figure 8B-C, large panels) while 5/5 mice inoculated with parental 4T1 contained >13,000 metastatic cells (Figure 8A, large panel). Primary tumor growth in immunocompetent syngeneic mice, therefore, is inconsistently reduced by expression of MHC class II or B7.1 genes, however, metastatic potential is reproducibly decreased.

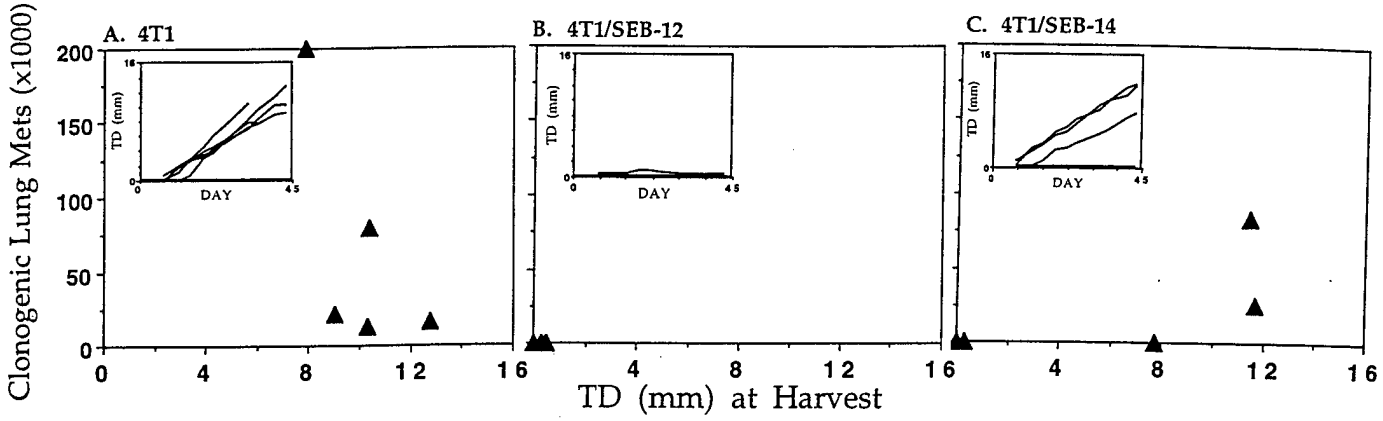


Figure 8: Expression of SEB reduces metastatic potential and tumorigenicity of the 4T1 transfectants. Female BALB/c mice were injected s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 cells (5 mice) (A), 4T1/SEB-12 (5 mice) (B), 4T1/SEB-14 (5 mice) (C), sacrificed 43 days later and the number of metastatic cells in the lungs determined. Primary tumors were measured every 3-4 days. Data are plotted as in Figure 6.

Tasks 8-9: *In vivo* tumor growth and spontaneous metastases development of 4T1 tumor double transfectants generated in task 3. As proposed in the SOW, these studies were projected to occur approximately during months 11-18. As expected, studies using the 4T1/A^{d}.B7.1 double transfectant are currently in progress. As other double transfectants become available, they will also be analyzed.

In summary, the *in vivo* analyses of the parental 4T1 tumor and the single transfectants have been completed, with the exception of the 4T1/4-1BB-L transfectants as they can not been screened.

Likewise, work is currently under way with the 4T1/A^d.B7.1 double transfectant. The 4T1 parental tumor appears to be an excellent model to study the effects of immunotherapy on metastatic breast cancer as our data shows that the pattern of metastatic spread is comparable to human mammary carcinoma. Assessment of the lung metastases will best approximate the extent of metastatic disease in tumor-bearing mice. More importantly, all of the single transfectants tested thus far have exhibited a decrease in metastatic potential and/or tumorigenicity as compared to parental 4T1.

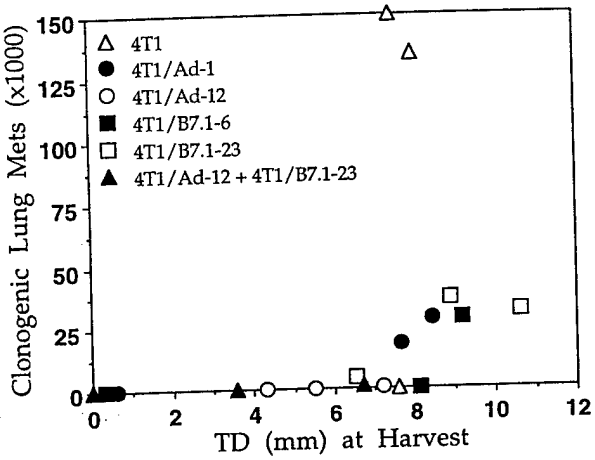
Technical Objective 3: Examine the ability of the immunogenic transfectants to prevent disease and/or eliminate established spontaneous metastases.

Task 10: Radiation sensitivity of 4T1 tumor cells. Before immunizations began, the radiation sensitivity of 4T1 was determined. Cells sustained increasing dosages of radiation ranging from 0 to 50,000 rads. Proliferation was measured 48 hours after exposure to radiation by using an MTT assay and was reduced by half at doses ranging 5000 to 10,000 rads. Final analysis was performed *in vivo*, as mice were injected i.p. with $1x10^6$ parental 4T1 cells exposed to 0, 5000, 8000, or 10000 rads. Average survival time for mice injected with non-irradiated 4T1 cells was 14 days, whereas the remaining animals lived long past 45 days. In the end, a dosage of 5000 rads was chosen for immunizations described in tasks 11-15.

Tasks 11-13: Therapy regimen one- Immunization of non-tumor bearing (naive) mice prior to challenge with parental tumor. The experiments in tasks 4-7 suggest that the reduced primary tumor and metastasis formation of the transfectants vs. 4T1 cells are due to increased tumor cell immunogenicity. As a result, we proposed 2 different regimens to analyze the efficacy of the transfectants as immunotherapeutic agents. In the first regimen, naive, tumor-free syngeneic BALB/c mice were immunized i.p. with 10^6 irradiated transfectants and challenged s.c. 4 weeks later with $5x10^3$ live 4T1 parental cells. Because the double transfectant was not available when these experiments were conducted, a mixture of 4T1/A^d and 4T1/B7.1 cells was used as one of the therapy groups. Mice were sacrificed 5 weeks after the 4T1 challenge and clonogenic tumor cells measured in the lungs. As shown in Figure 9, the class II^+ and $B7.1^+$ transfectants provided some protection against 4T1 metastasis, with 4T1/A^d-12 and the mixture of 4T1/A^d-12 plus 4T1/B7.1-23 providing

the maximum protection (<1,400 clonogenic cells in each individual lung), and immunization with wild type 4T1 providing minimal protection. Clonogenic metastatic cells in the liver and blood were also similarly reduced in the transfectant-treated animals (data not shown). Other organs were not monitored for metastatic cells. However, none of the transfectants significantly reduced the growth of the primary tumor (data not shown). Immunization of naive mice with the class II^+ and/or $B7.1^+$ transfectants significantly protects against spontaneous metastatic disease but does not affect primary tumor growth of wild type 4T1 tumor.

Figure 9: Immunization with MHC class II^+ or $B7.1^+$ transfectants protects naïve mice against metastatic disease from parental 4T1 tumor challenge. BALB/c mice (3 mice/group) were vaccinated i.p. with $1x10^6$ irradiated parental 4T1 (Δ) 41/A^d-1 (●), 4T1/A^d-12 (○), 4T1/B7.1-6 (■), 4T1/B7.1-23 (□), or a 1:1 mix of 4T1/A^d-12 + 4T1/B7.1-23 (▲). Four weeks later, mice were challenged s.c. in the abdominal mammary gland with $5x10^3$ live parental 4T1 cells Five weeks post-parental tumor challenge, the TD and clonogenic lung metastases were measured.



Tasks 14-15: Therapy regimen two- Treatment of mice bearing established 4T1 primary tumor

The second regimen was devised in order to model a more realistic clinical situation and to test the transfectants more rigorously. The therapeutic efficacy of two transfectant clones was further tested in mice against established metastases. BALB/c mice were challenged s.c. with $5x10^3$ wild type 4T1 tumor cells and, starting at either day 9 or 14 after 4T1 challenge, they were given injections of irradiated transfectants (4T1/A^d-12 and/or 4T1/B7.1-6) twice a week until the day of sacrifice, approximately 4 weeks later. At the time of sacrifice, primary tumor diameters of control-treated mice (ie. mice given irradiated 4T1 cells), 6.8-12.5 mm, were comparable to tumor diameters in

transfectant-treated animals, 6.3-13.6 mm. The two-tailed p value is 0.29 when comparing the tumor sizes of mice treated with control cells vs. transfectant-treated mice combined. Lungs were subsequently removed, and the number of clonogenic tumor cells determined. Since this therapy will be used to treat patients with established tumor, the results of this experiment have been plotted as number of clonogenic cells in the lungs vs. tumor diameter at the start of treatment. As shown in Figure 10, administration of 4T1/A^d-12, 4T1/B7.1-6, or a mixture of cells significantly reduces the number of lung metastases (panels B-D) relative to treatment with wild type 4T1 cells (panel A) when primary tumor diameters at the start of treatment were <4 mm. After transforming the number of clonogenic metastases to log values and analyzing as described in Materials and Methods, the two-tailed p value is 0.008 when comparing control-treated mice vs. transfectant-treated mice combined. When tumor diameters, however, were >4 mm on the initial treatment day, no significant

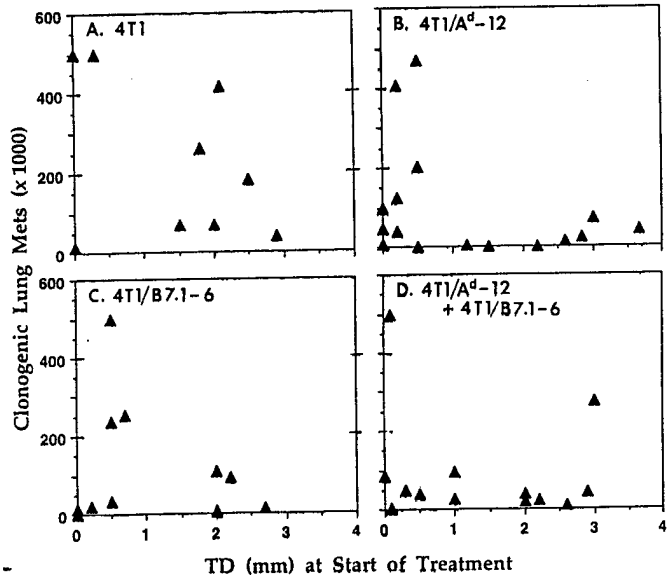


Figure 10: Immunotherapy of established 4T1 tumors with class II^+ and/or $B7.1^+$ transfectants reduces metastatic disease. BALB/c mice were challenged s.c. in the abdominal mammary gland with $5x10^3$ live parental 4T1 cells. At days 9 or 14 post-parental tumor challenge, TD was measured and therapeutic injections began. Mice were treated i.p. 2x a week, until the time of sacrifice, with $1x10^6$ irradiated parental 4T1 (A), 4T1/A^d-12 (B), 4T1/B7.1-6 (C), or a 1:1 mix of 4T1/A^d-12 plus 4T1/B7.1-6 (D) cells. Mice were sacrificed 6 weeks after initial 4T1 tumor challenge and the number of clonogenic lung metastases determined. The data are plotted as the TD at the time the therapeutic treatment began versus the number of clonogenic lung metastases (x 1000) at the time of sacrifice. Each triangle represents an individual mouse. Statistical analysis was performed using a student's t-test for unequal variances as described in the text (two-tailed p= 0.008).

reduction in primary tumor growth or metastatic cells was seen (data not shown). Metastatic spread, therefore, can be significantly reduced by immunotherapy in mice carrying spontaneously metastatic established tumors, provided treatment originates when the primary tumor is <4 mm in diameter.

Task 16: Analysis of immune response mechanism. To determine if T-cell-mediated immunity is involved in the reduced tumorigenicity and metastatic spread of the class II^+ and $B7.1^+$ transfectants described in tasks 6 and 7, T-cell deficient *nu/nu* mice were tumor challenged ($5x10^3$ cells) and followed for primary tumor growth and metastasis formation. Two MHC class II transfectants and two B7.1 transfectants were used. As shown in Figure 11, one of the class II^+ transfectants (4T1/A^d-1; panel B) and one of the $B7.1^+$ transfectants (4T1/B7.1-6; panel D) formed tumors and metastases in nude mice similar to unmodified wild type 4T1 tumor cells (panel A). In contrast, 4T1/A^d-12 (panel C) and 4T1/B7.1-23 (panel E) lines formed primary tumor comparable to 4T1, however their metastatic potential was much reduced relative to wild type 4T1 tumor cells. To analyze the effects of T-cells in immunocompetent vs. T-cell deficient mice, primary tumor incidence in BALB/c and BALB/c *nu/nu* mice were compared. As summarized in Table 3, 87% of the BALB/c *nu/nu* vs. 20% of the BALB/c mice developed progressive primary tumor following s.c. challenge. The class II^+ and $B7.1^+$ transfectants, therefore, have different primary growth kinetics and metastasis formation in T-cell deficient nude mice vs. immunocompetent BALB/c mice, suggesting that T-lymphocytes are important effector cells for regulating tumor growth *in vivo*.

Table 3: Tumor incidence of 4T1 transfectants in BALB/c vs. BALB/c *nu/nu* mice. Mice were challenged s.c. in the abdominal mammary gland with $5x10^3$ of transfected 4T1 tumor cells. The tumor incidence is the number of animals which developed progressive tumors. Animals were sacrificed when the TD reached 14-16 mm or when the mice became moribund.

Tumor Challenge	Tumor Incidence	
	BALB/c	BALB/c nu/nu
4T1/A^d-1	3/10	3/3
4T1/A^d-12	1/10	5/6
4T1/B7.1-6	2/5	7/8
4T1/B7.1-23	1/5	5/6

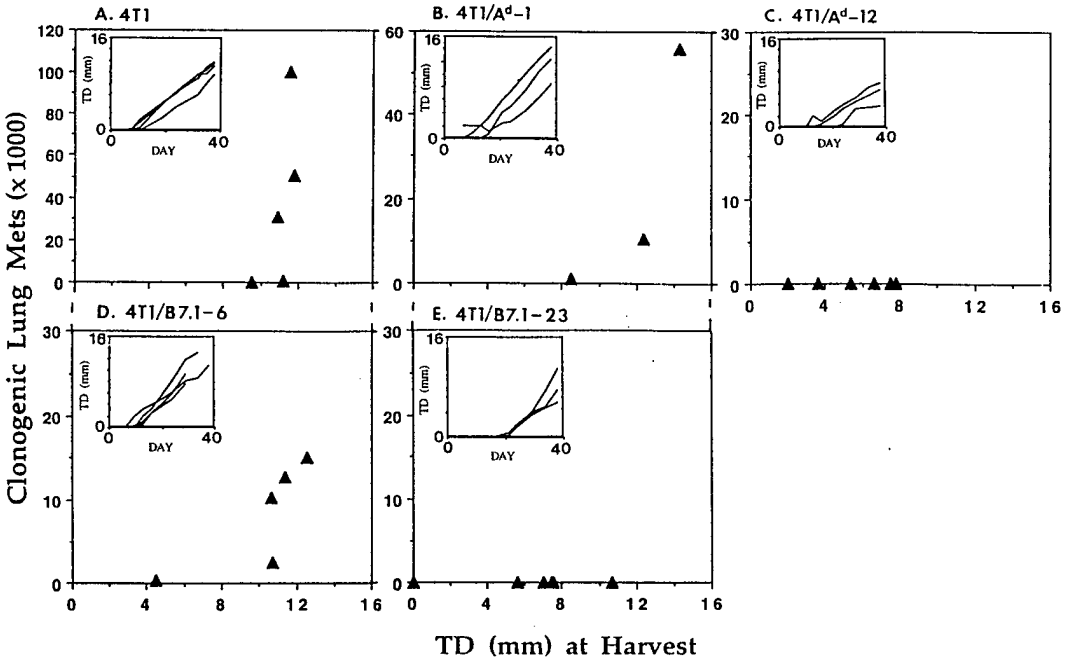


Figure 11: Different immune effector cells alter primary tumor growth versus spontaneous metastasis formation. BALB/c *nu/nu* mice were injected s.c. in the abdominal mammary gland with $5x10^3$ parental 4T1 (5 mice) (A), 4T1/A^d-1 (3 mice) (B), 4T1/A^d-12 (6 mice) (C), 4T1/B7.1-6 (5 mice) (D), or 4T1/B7.1-23 (6 mice) (E) and tumor growth measured every 3-4 days. Data are plotted as in Figure 6. Note that the number of clonogenic lung metastases shown on the y-axis ranges from 0-120 in panel A, as opposed to 0-60 for panel B and 0-30 for panels C-E.

In summary, experiments have begun which examine the ability of tumor cell-based vaccines to treat metastatic disease. The radiation dose required for immunizations has been determined as described in task 10. Both therapy regimens have been tested using the class II$^+$ and B7.1$^+$ transfectants and have shown promising results. It appears that our therapy will be effective in treating metastatic disease but not necessarily the primary tumor. Therapy experiments are currently in progress with the 4T1/SEB and 4T1/A^d.B7.1 transfectants and will be completed shortly. Although not extensively examined, studies to determine which effector cells are responsible for the enhanced immune response have begun. As described in task 16, nude mice were used to analyze the importance of T cells in the reduced tumorigenicity and metastatic potential observed in tasks 6 and 7 by the class II$^+$ and B7.1$^+$ transfectants. Ultimately, T cell depletion experiments are necessary to determine the role of effector cells in the therapy protocols described in tasks 11-15.

Conclusions

Many studies during the past 5-10 years have focussed on developing immunotherapy strategies for the treatment of solid tumors and have used animal systems to model human disease and to test the efficacy of immunotherapy. Most of these studies have used transplanted primary solid tumors (43, 44) or short-term established experimental (i.v. induced) metastatic cancers where therapy was performed very early during metastatic disease (45-48). A small number of studies focused on spontaneous metastases, however, these models used SCID mice or anatomically incorrect tumor challenge sites (49-51). In many cases, the growth characteristics and kinetics of the model tumors used do not closely follow the natural history of their corresponding human tumor and, hence, they are not optimal model systems. In contrast to many mouse tumors, the BALB/c-derived 4T1 mammary tumor, originally derived by F. Miller and colleagues (32-34), shares many characteristics with its human counterpart mammary carcinoma. For example, 4T1 spontaneously metastasizes while the primary tumor is in place, analogous to human mammary tumors. Sites of metastasis are common between the mouse and human malignancies: spreading first to the lungs and liver in 24-77% and 22-62% of women, respectively, vs. >95% and >75%, respectively, in BALB/c mice (Table 2) (52-55). Metastasis to the central nervous system is characteristically less frequent than metastasis to other sites in both humans and mice (30% and 40%, respectively) and statistically occurs later in the disease process (Table 2) (55, 56).

In addition to its growth characteristics, the 4T1 tumor has several experimental characteristics that make it an ideal model for testing immunotherapy strategies. A major asset is its stable resistance to 6-thioguanine, enabling the precise quantitation of very small numbers of tumor cells, long before they could be detected visually or accurately quantitated by other methods. Since metastasis to the lungs precedes and always accompanies metastasis to other organs (Table 2), quantitation of lung metastases accurately assesses metastatic disease. The similarity in growth between the 4T1 tumor and human mammary cancer, plus the ease of assessing metastatic disease, therefore, make the mouse 4T1 tumor an excellent model for testing potential immunotherapy strategies.

Previous immunotherapy studies using MHC class II and/or B7.1 expressing tumor cells as cell-based vaccines have dealt predominantly with solid, primary tumors (25, 26, 44). In our studies

these vaccines are used for the treatment of metastatic disease. They are also distinct from earlier studies using a variety of cell-based vaccines, including cytokine transduced/transfected tumor cells, in that spontaneous, established metastases are being treated, rather than short-term experimental (i.v.) metastases. These disease conditions, much more closely mimic those of human breast cancer patients, and hence the observed results may be useful in projecting experimental animal results to human clinical situations.

Treatment of mice carrying 9-14 day established 4T1 tumors with MHC class II and/or B7.1 transfected tumor cells results in a dramatic reduction in the number of metastatic tumor cells relative to mice treated with wild type 4T1 (Figure 10), suggesting that such cell-based vaccines may be useful immunotherapeutic agents for the treatment of metastases. The finding that metastatic growth is greatly reduced or eliminated, while primary tumor growth is not significantly impacted, is surprising, and suggests that immunotherapy may be more useful against metastatic disease than against primary tumor. Since many primary tumors can be successfully surgically resected while many metastatic lesions are refractile to current therapy, immunotherapy may have a unique role in cancer treatment.

Since mice with primary tumors of >2 mm contain lymph node and lung metastatic cells (Figure 6), the immunotherapy is limiting proliferation of pre-established metastases. Likewise, since treatment of naive mice produces some animals with no metastases, the immunotherapy is also preventing establishment of new metastases. Therefore, although not routinely curative, this immunotherapy may slow progression of metastatic disease.

Previous therapy studies with B7.1 transfected tumors and primary or experimental metastases indicated that costimulatory molecule expression was effective in vaccines containing "moderately" immunogenic tumor cells, but not in vaccines containing "poorly" immunogenic tumor cells (44). By definition, 4T1 cells are "poorly" immunogenic because immunization of tumor-free mice with irradiated wild type cells does not provide protective immunity against subsequent challenge with wild type tumor cells (Figures 9 and 10). Since immunization with B7.1 transfected tumor cells does not result in reduced primary tumor growth in the immunotherapy protocol, our results agree with these earlier studies (44). However, the finding that B7.1 transfected tumor cells promote significantly reduced metastatic growth in the therapy protocol (Figure 10), revives B7.1

as a potential candidate for immunotherapy.

The mechanism by which the class II^+ and $B7.1^+$ transfectants are providing their protection is not clear. As these transfectants displayed varying *in vivo* phenotypes, different types of effector cells may be activated. In most cases, T-cells were important in regulating primary tumor growth (Figure 11), however, their role in outgrowth of metastases is less clear cut. This could easily be explained by an enhancement of non-specific effectors such as LAK and/or NK, as it has been previously shown that B7.1 can induce NK activity against tumors (36, 57). Alternatively, limiting dilution cloning of the transfectants may have cloned out tumor cells which lost their ability to metastasize (58). Regardless of the *in vitro* and *in vivo* phenotypes of the transfectants (ie: level of expression of class II and/or B7.1, metastatic potential and tumorigenicity in BALB/c versus *nu/nu* mice) most clones provide some protection against wildtype metastatic disease (Figures 9 and 10). Thus, these studies suggest that most transfectants will be useful as vaccines and that cell-based vaccines may be more effective than previously thought.

Transfection of tumor cells with MHC class II plus B7.1 genes was originally designed to produce tumor cells that could directly present antigen to $CD4^+$ T_h cells and $CD8^+$ CTL and thereby facilitate optimal anti-tumor immunity (26, 59). Genetic experiments using bone marrow chimeras and sarcoma tumor cells support this hypothesized mechanism of $CD4^+$ T cell activation and demonstrate that the genetically modified tumor cells function as the antigen presenting cell (APC) for tumor-encoded antigen (60, 61). In contrast, class I-restricted tumor-encoded antigen appear to be presented indirectly via host-derived APC (62-64). Increased anti-tumor activity following immunization, therefore, is probably the result of enhanced presentation of tumor antigens and the subsequent activation of multiple helper and effector cell populations.

Why the effectiveness of this treatment is limited to mice with starting tumors of <4 mm is unclear. Factors such as immunosuppression of tumor-bearing individuals, immunogenicity of tumor antigens, the timing of the developing immune response versus outgrowth of the tumor, and involvement of non-specific effector cell types (ie: LAK, NK, macrophages) have been discussed at length in the context of other immunotherapy approaches (65-68) and some or all of these factors may be implicated here. Optimal T cell activation is achieved when B7.1 and MHC class II molecules are expressed by the same antigen presenting cell (26, 68). Our cell-based vaccine,

therefore, might be more effective if double-transfectants were used rather than the mixture of single transfectants tested in this study. Regardless of the limitations, however, the promising therapeutic responses are encouraging for further testing and development of this approach either alone, or in combination with other immunotherapeutic and/or conventional modalities.

Future Directions

Clearly the transfection of MHC class II and/or B7.1 alone are not sufficient to completely eliminate metastatic disease. More factors are needed to expand the immune response and extend successful treatment past a TD of 4 mm at the start of treatment. As discussed in this report, efforts are under way to add other potential mediators. However, another potential problem could be that the primary tumor is not removed in our system. As a result, a potential influx of metastatic cells caused by the primary tumor during therapy treatment exists. It is possible that while our therapy is eliminating the pre-existing metastatic disease the primary tumor continues to spread new metastases. Therefore, it is necessary to modify this proposal by developing this model further to include removal of the primary tumor before therapy.

References

1) Elliott, B.E., Carlow, D.A., Rodricks, A., & Wade, A. Perspectives on the role of MHC antigens in normal and malignant cell development. *Adv. Cancer Res.*, 53: 181-245, 1989.

2) Doyle, A., Martin, W.J., Funa, K., Gazdar, A., Carney, D., Martin, S.E., Linnoila, I.,Cuttitta, F., Mulshine, J., Bunn, P., & Minna, J. Markedly decreased expansion of class I histocompatibility antigens, protein, and mRNA in human small-cell lung cancer. *J. Exp. Med.*, 161: 1135-1151, 1985.

3) Lassam, N., & Jay, G. Suppression of MHC class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J. Immunol.*, 143: 3792-3797, 1989.

4) Kern, D., Klarnet, J., Jensen, M., & Greenberg, P. Requirement for recognition of class II molecules and processed tumor antigen for optimal generation of syngeneic tumor-specific class II-restricted CTL. *J. Immunol.*, 136: 4303-4310, 1986.

5) Hood, L., Steinmetz, M., & Malissen, B. Genes of the major histocompatibility complex of the mouse. *Ann. Rev. Immunol.*, 1: 529-568, 1983.

6) Lancki, D.W., Ma, D.I., Havran, W.L., & Fitch, F.W. Cell surface structures involved in T cell activation. *Immunol. Rev.*, 81: 65-94, 1984.

7) Chen, L., Ashe, S., Brady, W.A., Hellstrom, I., Hellstrom, K.E., Ledbetter, J.A., Mc Gowan, P., & Linsley, P.S. Costimulation of antitumor immunity by the B7 counterreceptor for the T-lymphocyte molecules CD28 and CTLA-4. *Cell*, 71: 1093-1102, 1992.

8) Miller, J.F.A.P., & Heath, W.R. Self-ignorance in the peripheral T-cell pool. *Immunol. Rev.*, 133: 131-150, 1993.

9) Colombo, M., & Forni, G. Cytokine gene transfer in tumor inhibition and tumor therapy: Where are we now? *Immunol. Today*, 15: 48-51, 1994.

10) Pardoll, D.M. Cancer vaccines. *Immunol. Today*, 14: 310-316, 1993.

11) Asher, A.L., Mule, J.J., Kasid, A., Restifo, N.P., Salo, J.C., Reichert, C.M., Jaffe, G., Fendly, B., Kreigler, M., & Rosenberg, S.A. Murine tumor cells transduced with the gene for tumor necrosis factor-α: Evidence for paracrine immune effects of tumor necrosis factor against tumors. *J. Immunol.*, 146: 3227-3234, 1991.

12) Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., & Mulligan, R.C. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*, 90: 3539-3543, 1993.

13) Pulaski, B.A., Mc Adam, A.J., Hutter, E.K., Biggar, S., Lord, E.M., & Frelinger, J.G. Interleukin 3 enhances development of tumor-reactive cytotoxic cells by a CD4-dependent mechanism.*Cancer Res.*, 53: 2112-2117, 1993.

14) Collins, T.L., Kassner, P.D., Beirer, B.E., & Burakoff, S.J. Adhesion receptors in lymphocyte activation. *Curr. Opin. Immunol.*, 6: 385-393, 1994.

15) Janeway, C.A. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Ann. Rev. Immunol.*, 10: 645-674, 1992.

16) Macatonia, S.E., Taylor, P.M., Knight, S.C., & Askonas, B.A. Pimary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses *in vitro*. *J. Exp. Med.*, 169: 1255-1264, 1989.

17) Allison, J.P. CD28-B7 interactions in T-cell activation. *Curr. Opin. Immunol.*, 6: 414-419, 1994.

18) Liu, Y., Jones, B., Aruffo, A., Sullivan, K.M., Linsley, P.S., and Janeway Jr., C.A. Heat-stable antigen is a co-stimulatory molecule for CD4 T cell growth. *J. Exp. Med.*, 175: 437-445, 1992.

19) Liu, Y., Jones, B., Brady, W., Janeway Jr., C.A., and Linsley, P.S. Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. *Eur. J. Immunol.*, 22: 2855-2859, 1992.

20) Damle, N.K., Klussman, K., Linsley, P.S., and Aruffo, A. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and V-CAM-1 on resting and antigen-primed $CD4^+$ lymphocytes. *J. Immunol.*, 148: 1985-1992, 1992.

21) Goodwin, R.G., Din, W.S., Davis-Smith, T., Anderson, D.M., Gimpel, S.D., Sato, T.A., Maliszewski, C.R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., Farrah, T., Armitage, R.J., Fanslow, W.C., and Smith, C.A. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur.*

J. Immunol., 23: 2631-2641, 1993.

22) DeBenedette, M.A., Chu, N.R., Pollok, K.E., Hurtado, J., Wade, W.F., Kwon, B.S., and Watts, T.H. Role of 4-1BB- ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP. *J. Exp. Med.*, 181: 985-992, 1995.

23) Pollock, K.E., Kim, Y.-J., Hurtado, J., Zhou, Z., Kim, K.K., and Kwon, B.S. 4-1BB T-cell antigen binds to mature B cells and macrophages, and costimulates anti-μ-primed splenic B cells. *Eur. J. Immunol.*, 24: 367-374, 1994.

24) Hurtado, J., Kim, S.H., Pollock, K.E., Lee, Z.H., and Kwon, B.S. Potential role of 4-1BB in T-cell activation: comparison with the costimulatory molecule CD28. *J. Immunol.*, 155: 3360-3367, 1995.

25) Ostrand-Rosenberg, S., Baskar, S., Patterson, N., and Clements, V. Expression of MHC class II and B7-1 and B7-2 costimulatory molecules accompanies tumor rejection and reduces the metastatic potential of tumor cells. *Tissue Antigens*, 47: 414-421,1996.

26) Baskar, S., Gilmcher, L., Nabavi, N., and Ostrand-Rosenberg, S. MHC class class $II^{+}B7\text{-}1^{+}$ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. *J. Exp. Med.*, 181: 619-628, 1995.

27) Herman, A., Kappler, J.W., Marrack, P., and Pullen, A.M. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Ann. Rev. Immunol.*, 9: 745-772, 1991.

28) Marrack, P., Blackman, M., Kushnir, E., and Kappler, J. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J. Exp. Med.*, 171: 455-464, 1990.

29) Newell, K.A., Ellenhorn, J.D.I., Bruce, D.S., and Bluestone, J.A. *In vivo* T-cell activation by staphylococcal enterotoxin B prevents outgrowth of a malignant tumor. *Proc. Natl. Acad. Sci., USA*, 88: 1074-1078, 1991.

30) Shu, S., Krinock, R.A., Matsumura, T., Sussman, J.J., Fox, B.A., Chang, A.E., and Terman, D.S. Stimulation of tumor-draining lymph node cells with superantigenic staphylococcal toxins leads to the generation of tumor-specific effector T cells. *J. Immunol.*, 152: 1277-1288, 1994.

31) Penna, C., Dean, P.A., and Nelson, H. Antitumor x anti-CD3 bifunctional antibodies redirect T-cells activated *in vivo* with staphylococcal enterotoxin B to neutralize pulmonary metastases. *Cancer Res.*, 54: 2738-2743, 1994.

32) Dexter, D.L., Kowalski, H.M., Blazar, B.A., Fligiel, Z., Vogel, R., and Heppner, G.H. Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res.*, 38: 3174-3181, 1978.

33) Miller, F.R., Miller, B.E., and Heppner, G.H. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. *Invasion Metastasis*, 3: 22-31, 1983.

34) Aslakson, C.J., and Miller, F.R. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res.*, 52: 1399-1405, 1992.

35) Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y., & Kedes, L. A human β-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA*, 84: 4831-4835, 1987.

36) Yeh, K-Y., Pulaski, B.A., Woods, M.L., McAdam, A.J., Gaspari, A.A., Frelinger, J.G., and Lord, E.M. B7-1 enhances natural killer cell-mediated cytotoxicity and inhibits tumor growth of a murine lung adenocarcinoma. *Cell. Immunol.*, 165: 217-224, 1995.

37) Ozato, K., Mayer, N.M., and Sachs, D.H. Monoclonal antibodies to mouse major histocompatibility complex antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. Transplantation, *34:* 113-118, 1982.

38) Ozato, K., Mayer, N.M., and Sachs, D.H. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J. Immunol., *124:* 533-540, 1980.

39) Kappler, J.W., Skidmore, B., White, J., and Marrack, P. Antigen-inducible, H-2-restricted, IL-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med., *153:* 1198-1214, 1981.

40) Janeway, C.A. Jr., Conrad, P.J., Lerner, E.A., Babich, J., Wettstein, P., and Murphy, D.B. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cellbound Ia antigens as targets of immunoregulatory T cells. J. Immunol., *132:* 662-667, 1984.

41) Nabavi, N., Freeman, G.J., Gault, A., Godfrey, D., Nadler, L.M., & Glimcher, L.M.

Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. Nature, *360:* 266-268, 1992.

42) Pulaski, B.A., and Ostrand-Rosenberg, S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Res.*, 58: 1486-1493, 1998.

43) Blankenstein, T., Cayeux, S., and Qin, Z. Genetic approaches to cancer immunotherapy. Rev. Phys. Biochem. Pharm., *129:* 1-49, 1996.

44) Hellstrom, K.E., Hellstrom, I., and Chen, L. Can co-stimulated tumor immunity be therapeutically efficacious? Immunolog. Rev., *145:* 123-145, 1995.

45) Li, Y., Hellstrom, E.K., Newby, S.A., and Chen, L. Costimulation by CD48 and B7-1 induces immunity against poorly immunogenic tumors. J. Exp. Med., *183:* 639-644, 1996.

46) Rodolfo, M, Zilocchi, C., Melani, C., Cappetti, B., Arioli, I., Parmiani, G., and Colombo, M.P. Immunotherapy of experimental metastases by vaccination with interleukin gene-transduced adenocarcinoma cells sharing tumor-asssociated antigens. Comparison between IL-12 and IL-2 gene-transduced tumor cell vaccines. J. Immunol., *157:* 5536-5542, 1996.

47) Chamberlain, R.S., Carroll, M.W., Bronte, V., Hwu, P., Warren, S., Yang, J.C., Nishimura, M., Moss, B., Rosenberg, S.A., and Restifo, N.P. Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. Cancer Res., *56:* 2832-2836, 1996.

48) Zitvogel, L., Tahara, H., Robbins, P.D., Storkus, W.J., Clarke, M.R., Nalesnik, M.A., and Lotze, M.T. Cancer immunotherapy of established tumors with IL-12. Effective delivery by genetically engineered fibroblasts. J. Immunol., *155:* 1393-1403, 1995.

49) Zheng, L.M., Ojcius, D.M., Garaud, F., Roth, C., Maxwell, E., Li, Z., Rong, H., Chen, J., Wang, X.Y., Catino, J.J., and King, I. Interleukin-10 inhibits tumor metastases through an NK cell-dependent mechanism. J. Exp. Med., *184:* 579-584, 1996.

50) Coveney, E., Clary, B., Iacobucci, M., Philip, R., and Lyerly, K. Active immunotherapy with transiently transfected cytokine-secreting tumor cells inhibits breast cancer metastases in tumor-bearing animals. Surgery, *120:* 265-272, 1996.

51) Porgador, A., Tzehoval, E., Vadai, E., Feldman, M., and Eisenbach, L. Combined vaccination with major histocompatibility class I and interleukin 2 gene-transduced melanoma

cells synergizes the cure of postsurgical established lung metastases. Cancer Res., *55:* 4941-4149, 1995.

52) Rutgers, E.J.Th., van Slooten, E.A., and Kluck, H.M. Follow-up treatment of primary breast cancer. Br. J. Surg., *76:* 187-190, 1989.

53) Tomin, R., and Donegan, W.L. Screening for recurrent breast cancer- its effectiveness and prognostic value. J. Clin. Oncol., *5:* 62-67, 1987.

54) Kamby, C., Dirksen, H., Vejborg, I., Daugaard, S., Guldhammer, B., Rossing, N., and Mouridsen, H.T. Incidence and methodologic aspects of the occurrence of liver metastases in recurrent breast cancer. Cancer, *59:* 1524-1529, 1987.

55) Amer, M.H. Chemotherapy and pattern of metastases in breast cancer patients. J. Surg. Oncol., *19:* 101-105, 1982.

56) Boogerd, W. Central nervous system metastasis in breast cancer. Radiother. Oncol., *40:* 5-22, 1996.

57) Geldhof, A.B., Raes, G., Bakkus, M., Devos, S., Thielemans, K., and DeBaetselier, P. Expression of B7-1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity. Can. Res., *55:* 2730-2733, 1995.

58) Hart, I.R., and Fidler, I..J. The implications of tumor heterogeneity for studies on the biology and therapy of cancer metastases. Biochim. Biophys. Acta, *651:* 37-50, 1981.

59) Ostrand-Rosenberg, S., Thakur, A., and Clements, V. Rejection of mouse sarcoma cells after transfection of MHC class II genes. J. Immunol., *144:* 4068-4071, 1990.

60) Armstrong, T.D., Clements, V., and Ostrand-Rosenberg, S. MHC class II-transfected tumor cells directly present antigen to tumor-specific $CD4^+$ T lymphocytes. J. Immunol., *160:* 661-666, 1998.

61) Armstrong, T.D., Pulaski, B.A., and Ostrand-Rosenberg, S. Tumor antigen presentation: changing the rules. Cancer Immunol. Immunother., (in press).

62) Huang, A., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., Levitsky, H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science, *264:* 961-965, 1995.

63) Huang, A., Bruce, A., Pardoll, D., Levitsky, H. Does B7-1 expression confer antigen-

presenting cell capacity to tumors *in vivo*? J. Exp. Med., *183:* 769-776, 1996.

64) Pulaski, B.A., Yeh, K.-Y., Shastri, N., Maltby, K.M., Penney, D., Lord, E., and Frelinger, J.G. IL-3 enhances CTL development and class I MHC presentation of exogenous antigen by tumor-infiltrating macrophages. Proc. Natl. Acad. Sci. USA, *93:* 3669-3674, 1996.

65) Levey, D.L., and Srivastava, P.K. Alterations in T cells of cancer-bearers: whence specificity? Immunol. Today, *17:* 365-368, 1996.

66) Rosenberg, S.A. Cancer vaccines based on the identification of genes encoding cancer regression antigens. Immunol. Today, *18:* 175-182, 1997.

67) Van den Eynde, B.J., and van der Bruggen, P. T cell-defined tumor antigens. Curr. Opin. Immunol., in press, 1997.

68) Liu, Y., and Janeway, C. Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. Proc. Natl. Acad. Sci. USA, *89:* 3845-3849, 1992.